A new method for determination of ascorbic acid in fruit juices, pharmaceuticals and biological samples

Etesh K Janghel*, Santosh Sar† and Y Pervez‡

1Department of Applied Chemistry, Ashoka Institute of Technology & Management, Gram- Torankatta, Post-Somni, Rajnandgaon 491 441, India
2Bhilai Institute of Technology, Bhilai House, Durg 491 001, India
3Chhatrapati Shivaji Institute of Technology, Balod Road, Durg 491 001, India

Received 23 March 2012; revised 22 June 2012; accepted 28 June 2012

This study presents a new, selective and accurate indirect spectrophotometric method for determination of L-ascorbic acid in fruit juices, pharmaceuticals and biological samples. Beer’s law is obeyed by ascorbic acid (conc. range, 0.8-6.3 µg) per 25 ml of final solution (0.032-0.252 ppm). The method showed: apparent molar absorptivity, 1.56 × 10^5 l mol⁻¹ cm⁻¹; Sandell’s sensitivity, 0.0024 µg cm⁻²; limit of detection, 0.0086 µg ml⁻¹; and limit of quantitation, 0.01 µg ml⁻¹. It was satisfactorily applied for determination of ascorbic acid in fruit juices, pharmaceuticals and biological samples. Reliability of method was established by parallel determination against Leuco crystal violet and Rhodamine – B method.

Keywords: Ascorbic acid, Auramine O dyes, Biological Samples, Pharmaceuticals, Spectrophotometry

Introduction

Ascorbic acid (AA; also known as L-ascorbic acid, antiscorbutic vitamin and vitamin C) is an important water soluble vitamin. Humans and apes cannot synthesize ascorbic acid due to lack of gulonolactone oxidase enzyme, and hence ascorbic acid has to be supplemented from external sources, mainly through fruits, vegetables and tablets. Major metabolites of AA in human body are dehydroascorbic acid, 2,3-diketogluconic acid and oxalic acid. Vitamin C is one of the most essential vitamins for both pharmaceutical and food processing industries in view of its nutritional significance, varied uses in food and high daily doses necessary for optimum health. A large number of methods for determination of AA include titrimetry, voltammetry, amperometry, conductometry, potentiometry, fluorometry, chemiluminescence, flow-injection analysis (FIA) and chromatography. Among spectrophotometric determination of AA, formation of an osazone (bis-(2,4-dinitrophenyl)hydrazone) derivative of AA is a complex, time consuming method and subject to several interferences. Modifications have been suggested improving this method. The method using 2,6-dichlorophenol indophenols sodium (DCPIP) is subject to several limitations. Silver-gelatin complex has been used in reductive spectrophotometric method. Oxidation of AA with Fe(III) and complexation of resulting Fe(II) with 1,10-phenonthonline, besides a simple kinetic spectrophotometric measurement of AA based on the reduction of toluidine blue are reported. An indirect spectrophotometric determination of AA based on extraction of iodine produced by reduction of potassium iodate is also reported. Many other reagents [fast red, leuco crystal violet, methyl viologen, Leuco malachite green, rhodamine-B, Cu(II) – EDTA complex, 2 – mercaptoethanol and reverse flow injection analysis (rFIA) etc.] have also been used in determination of AA.

This study presents a simple method for determination of AA using auramine O as chromogenic reagent. Proposed method was successfully applied for determination of AA in fruit juices, pharmaceutical and biological samples.

Experimental Section

All chemicals used were of Anal R grade. Double distilled deionized water was used. For ascorbic acid (Loba Chemie), a stock solution (1 mg/ml) was prepared.
by dissolving 100 mg of AA in 100 ml in water. Working
standard solutions were freshly prepared by appropriate
dilution of stock solutions with water. Other solutions
were used as aqueous solution of the following: auramine
O (Stuttgart, W. Germany), 0.01%; sodium acetate,
2.0-mol l$^{-1}$; oxalic acid, 0.2-mol l$^{-1}$; metaphosphoric acid,
3%; sodium salt of EDTA, 5%; potassium iodide (Merck),
0.1-mol l$^{-1}$; potassium iodate (Merck), 0.2 mol l$^{-1}$; and
hydrochloric acid (HCl), 0.02-mol l$^{-1}$. Potassium iodide-
potassium iodate (KI-KIA) mixture was prepared by
mixing 0.1-mol l$^{-1}$ potassium iodide and 0.2-mol l$^{-1}$
potassium iodate in 5:1 ratio. This solution was prepared
fresh daily and kept in amber coloured bottle. A Systronic
UV – Vis spectrophotometer 108 with 2 cm matched
silica cells was used for all spectral measurements. pH
meter model 331 was used for pH measurements. A
Remi C- 854/4 clinical centrifugal having a maximum
centrifugal force of 1850 g with fixed swing out rotors
was used for centrifugation.

**Procedure for Determination of Ascorbic Acid (AA)**

An aliquot of sample solution (AA, 0.8-6.3 µg) was
transferred into a series of 25 ml graduated tube. To
this, (KI-KIA) mixture solution (0.4 ml) and 0.02-mol l$^{-1}$
HCl (1 ml) were added, and mixture was gently shaken
until appearance of yellow colour, indicating liberation of
iodine. Then, 1 ml of 0.01% auramine O dye solution
was added, followed by addition of 2 ml of 2.0-mol l$^{-1}$
sodium acetate, and reaction mixture was shaken for
2 min. The mixture was diluted up to 25 ml with distilled
water and mixed well (Fig. 1). It was kept for 10-15 min
for completion of reaction. Absorbance of resulting
solution was measured at 405 nm against distilled water.
A blank was prepared by replacing AA solution with
distilled water. Absorbance corresponding to bleached
color, which in turn corresponds to AA concentration,
was obtained by subtracting absorbance of blank solution
from that of test solution.

**Table 1—Determination of ascorbic acid in fruits and vegetables (Results of analysis of real samples and recovery from spiked samples)**

<table>
<thead>
<tr>
<th>Samples*</th>
<th>Ascorbic acid originally found$^a$</th>
<th>Ascorbic acid added, µg</th>
<th>Total ascorbic acid by proposed method (c)</th>
<th>Difference (c-a)</th>
<th>Recovery%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proposed method, µg</td>
<td>Reported method$^{1,30}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(a)</td>
<td>µg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Orange</td>
<td>5.39</td>
<td>5.23</td>
<td>3.0</td>
<td>8.23</td>
<td>2.84</td>
</tr>
<tr>
<td>Lemons</td>
<td>5.92</td>
<td>5.85</td>
<td>3.0</td>
<td>8.90</td>
<td>2.98</td>
</tr>
<tr>
<td>Potatoes</td>
<td>3.97</td>
<td>3.92</td>
<td>2.0</td>
<td>5.86</td>
<td>1.89</td>
</tr>
<tr>
<td>Chillies</td>
<td>2.93</td>
<td>2.78</td>
<td>2.0</td>
<td>4.83</td>
<td>1.90</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>2.93</td>
<td>2.78</td>
<td>2.0</td>
<td>4.91</td>
<td>1.98</td>
</tr>
</tbody>
</table>

*$^a$Aliquot of samples = 1 ml; $^{**}$Mean of 5 replicate analysis

**Determination of Ascorbic Acid (AA) in Fruit Juices and Vegetables**

Fruit samples (orange, lemon; 5 g each) were
weighed; juice was separated from fruits with a
mechanical press and centrifuged. A 1.0 ml aliquot of
juice was diluted to 100 ml with 0.2 mol/l oxalic acid to
avoid losses of AA due to air oxidation, To this mixture,
5% EDTA (1 ml) was added and the solution was
centrifuged at 1850 g for 5 min. Supernatant liquid
was further diluted to a suitable volume with deionized water
on the basis of AA concentration in fruits. In aliquot
(1 ml), auramine O dye (2 ml) was added, followed by
sodium hydroxide (2 ml) and then analyzed (Table 1).
Various samples of vegetables (potatoes, chillies and grapefruit) were cut into small pieces and 4-5 g were homogenized with 100-150 ml of 0.2 mol/l oxalic acid as soon as possible to avoid any oxidation of AA. To this, added 1 ml of 5% EDTA and centrifuged. Supernatant liquid was diluted to a suitable volume and 1 ml aliquot was analyzed (Table 1).

### Determination of Ascorbic Acid (AA) in Pharmaceuticals and Biological Samples

All drug samples tested were fresh and purchased from local pharmacy. An AA tablet or content of a capsule was weighed, ground to a fine powder and stirred for 2-3 min with 50 ml of deionized water. Then 5% EDTA (1 ml) was added and filtered through Whatman No. 41 filter paper. Insoluble mass was washed with three successive 5 ml portions of water and filtrate plus washings were diluted to 250 ml calibrated flask. A known volume was further diluted depending on AA content and colour of the sample. Aliquot (1 ml) was analyzed (Table 2). Since presence of AA has been reported in human milk, blood and urine samples, this method was applied for AA determination in these samples. Prior to determination of AA, 5% EDTA (1 ml) and 1% TCA (2 ml) were added, centrifuged, diluted to a suitable volume and aliquot (1 ml) was analyzed (Table 3).

### Result and Discussion

#### Absorption Spectra

Reaction of AA with (KI-KIA) mixture in acidic medium liberated iodine, which selectively bleached brown colour of auramine O dye, thereby decreasing dye absorbance at 405 nm (Fig. 2). Decrease in absorbance is directly proportional to AA concentration. Reagent blank showed maximum absorbance at this wavelength.

---

### Table 2—Results of determination of ascorbic acid contents in pharmaceutical products

<table>
<thead>
<tr>
<th>Vitamin C tablets</th>
<th>Proposed method</th>
<th>Reported method</th>
<th>Claimed value</th>
<th>Reported reference method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brand – A</td>
<td>99.90 (± 0.2)</td>
<td>99.78 (± 0.2)</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>Brand – A</td>
<td>99.92 (± 0.2)</td>
<td>99.85 (± 0.092)</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Brand – B</td>
<td>499.89 (± 0.1)</td>
<td>499.80 (± 0.1)</td>
<td>500</td>
<td>3</td>
</tr>
<tr>
<td>Brand – B</td>
<td>499.95 (± 0.3)</td>
<td>499.92 (± 0.019)</td>
<td>500</td>
<td>30</td>
</tr>
</tbody>
</table>

### Table 3—Results of determination of ascorbic acid in milk, blood and urine sample

<table>
<thead>
<tr>
<th>Samples*</th>
<th>Ascorbic acid originally found*</th>
<th>Ascorbic acid added µg (b)</th>
<th>Total ascorbic acid by proposed method (c)</th>
<th>Difference (c-a)</th>
<th>Recovery% (c-a) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk sample°,30</td>
<td>2.56 (a) 2.65 (b)</td>
<td>2.0</td>
<td>4.53</td>
<td>1.97</td>
<td>98.5</td>
</tr>
<tr>
<td></td>
<td>1.67 (a) 1.53 (b)</td>
<td>2.0</td>
<td>3.64</td>
<td>1.97</td>
<td>98.5</td>
</tr>
<tr>
<td>Blood sample°,30</td>
<td>3.66 (a) 3.48 (b)</td>
<td>1.5</td>
<td>5.02</td>
<td>1.36</td>
<td>90.6</td>
</tr>
<tr>
<td></td>
<td>1.75 (a) 1.39 (b)</td>
<td>2.0</td>
<td>3.68</td>
<td>1.93</td>
<td>96.5</td>
</tr>
<tr>
<td>Urine°</td>
<td>2.36 (a) 2.48 (b)</td>
<td>2.0</td>
<td>4.24</td>
<td>1.88</td>
<td>94.0</td>
</tr>
</tbody>
</table>

*Amount of samples = 2 ml; **Mean of 5 replicate analysis
Beer’s Law and Sensitivity

Plotting absorbance vs concentration of AA gave straight-line. Beer’s law was obeyed in AA (conc., 0.8-6.3 µg) per 25 ml of final solution. The method showed: apparent molar absorptivity, $1.56 \times 10^5$ l mol$^{-1}$cm$^{-1}$; Sandell’s sensitivity, 0.0024 µg cm$^{-2}$; limit of detection (LOD), 0.0086 µg ml$^{-1}$; and limit of quantitation (LOQ), 0.01 µg ml$^{-1}$. Reproducibility of method was checked by 5 replicate measurements, each containing 4.0 µg AA per 25 ml of final solution. Standard deviation (SD) and relative standard deviation (RSD) were ± 0.0025 and ±0.9%, respectively. Lower RSD (±0.9 %) and range of error at 92% confidence level in terms of absorbance were ± 0.003, indicating good precision of the method.

Conditions for Colour Development

Constant and maximum difference in absorbance was obtained when KI-KIA mixture (5:1) solution (0.4 ml), 0.02-mol l$^{-1}$ HCl (1 ml), 0.01% auramine O dye (1 ml) and 2.0 mol l$^{-1}$ sodium acetate (2.0 ml) were added to get maximum sensitivity. By increasing KI-KIA mixture amount, absorbance remained constant. A series of buffer solutions differing by pH 0.5 were prepared. Constant and maximum difference in absorbance values were obtained at pH= 4±0.2, (maintained throughout the study by acetate buffer). Under optimum conditions, constant absorbance values were obtained instantaneously and required no heating. Therefore, determination was carried out at room temperature (RT: 30°C) and time taken for completion of reaction was 10-15 min (Fig. 3). Bleached colour was stable for more than 24 h.

Interference Studies

Method validity was assessed by investigating tolerance limit value of different foreign species in a
solution containing 5.0 µg per 100 ml of AA (Table 4). Species (folic acid, ferrous fumarate vitamins B₁, B₂, B₆, B₁₂, nicotinamide and calcium pentothenate) that are commonly present in pharmaceutical preparations and a number of organic acids that are known to be present in fresh fruit juices do not interfere with the proposed method. Interference of metal ions (Fe³⁺ & Al³⁺) was prevented by addition of 5% EDTA (1 ml) prior to the addition of solution hydroxide solution.

### Table 4—Effect of foreign species (Ascorbic acid conc., 5.0 µg in 25 ml solution)

<table>
<thead>
<tr>
<th>Foreign species</th>
<th>Tolerance limit* µg ml⁻¹</th>
<th>Foreign species</th>
<th>Tolerance limit* µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺, glucose, sucrose, citric acid</td>
<td>3200</td>
<td>Nicotinamide</td>
<td>4500</td>
</tr>
<tr>
<td>SO₄²⁻, PO₄³⁻</td>
<td>2600</td>
<td>Ferrous fumarate</td>
<td>4250</td>
</tr>
<tr>
<td>Tartaric acid, malic acid</td>
<td>900</td>
<td>Calcium pentothenate</td>
<td>1150</td>
</tr>
<tr>
<td>Al³⁺, Fe³⁺, Zn²⁺</td>
<td>800</td>
<td>Vitamins</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>B₁, B₂</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B₆</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B₁₂</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Folic acid</td>
<td>50</td>
</tr>
</tbody>
</table>

*Amount of foreign species causing ± 2% in absorbance values

### Table 5—Comparison of proposed (present) method with other spectrophotometric methods

<table>
<thead>
<tr>
<th>Reagents</th>
<th>λmax nm</th>
<th>Beers law range/ Detection limit, µg ml⁻¹</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 4 –d-Dinitrophenyl hydrazine (II)²⁰</td>
<td>524</td>
<td>8</td>
<td>Only applicable to biological materials, require long waiting time, less sensitive</td>
</tr>
<tr>
<td>2, 6 – Dichlorophenol indophenol sodium EDTA²²</td>
<td>520</td>
<td>1</td>
<td>Subject to limitations arising from its poor specificity and dye stability, serious interference of molecule or ions, which reduce DCPIP</td>
</tr>
<tr>
<td>Cu(II) – ED + A-complex³¹</td>
<td>267</td>
<td>0.20</td>
<td>Less sensitive and selective, not applicable to beverages, in which matrices absorb or scatter strongly in UV region</td>
</tr>
<tr>
<td>Silver gelatin complex³³</td>
<td>415</td>
<td>1-10</td>
<td>Use of expensive reagent less sensitive</td>
</tr>
<tr>
<td>Reduction of potassium iodate²⁴</td>
<td>520</td>
<td>0.20 - 0.36</td>
<td>Less sensitive</td>
</tr>
<tr>
<td>2 – Mercaptoethanol³²</td>
<td>245</td>
<td>0.20</td>
<td>Instrumental method using FIA technique</td>
</tr>
<tr>
<td>Methyl viologen³</td>
<td>600</td>
<td>0.1 – 1.0</td>
<td>Highly sensitive and selective, colour stability 2 h</td>
</tr>
<tr>
<td>Rhodamine-B³⁰</td>
<td>555</td>
<td>0.048-0.48</td>
<td>Less sensitive</td>
</tr>
<tr>
<td>Tris, 1, 10- phenononthroline</td>
<td>510</td>
<td>50-400</td>
<td>Applied for determination to wide range and less Complex³³ sensitive</td>
</tr>
<tr>
<td>Fast red³⁷</td>
<td>630</td>
<td>5.25</td>
<td>Colour is stable for only 2 h and less sensitive</td>
</tr>
<tr>
<td>Leucocrystal violet²⁸</td>
<td>590</td>
<td>0.01-0.1</td>
<td>Though method is highly sensitive but the reagent used is costly and not easily available</td>
</tr>
<tr>
<td>Leuco malachite green³⁹</td>
<td>620</td>
<td>0.032-0.32</td>
<td>Less sensitive</td>
</tr>
<tr>
<td>p-aminoacetophenon³⁹</td>
<td>460</td>
<td>0.4-6.5</td>
<td>Instrumental method using FIA technique</td>
</tr>
<tr>
<td>Auramine O(Present method)</td>
<td>405</td>
<td>0.032- 0.25</td>
<td>Simple, highly sensitive, cost effective, higher stability of colour, no need of heating and extraction into organic phase and applicable to wide range of samples</td>
</tr>
</tbody>
</table>

### Application

The method has been applied satisfactorily to determination of AA in fruit juices, vegetables, pharmaceuticals, milk, blood and urine, and results obtained (Tables 1-3) are in good agreement with reported methods. The results of pharmaceuticals obtained by proposed method also agreed well with the claimed value on labels in all instances. To check validity of the method, known amounts of AA were added to various samples.
of fruits, vegetables, milk, blood and urine and then analysed by proposed as well as reported methods. Recoveries were found to be ~ 73-99% (Table 5), which is close to the results of established methods, indicating accuracy of the proposed method.

Conclusions
This study provided a new, simple and highly sensitive method for determination of AA in fruit juices, pharmaceutical and biological samples. Sensitivity in terms of molar absorbptivity and precision in terms of RSD indicated that proposed method is very reliable for determination of AA in various samples. The method needed neither heating nor extraction in organic phase. This method is good alternative to some reported costly instrumental method and its advantages were mainly due to its cheaper cost, easier availability and higher stability of colour of auramine O dye.

Acknowledgments
Authors thank Principal and Head, Chhatrapati Shivaji Institute of Technology, Durg, Principal & Head, Ashoka institute of Technology & Management, Rajnandgaon, for providing laboratory facilities and financial assistance.

References


